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Overcoming physiological seed dormancy in semi-arid *Prostanthera* (Labiatae)

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Abstract

The genus *Prostanthera* belongs to the family Labiatae, and is comprised of about 100 species endemic to Australia. With the exception of a few species that have previously been studied, the dormancy mechanism and germination requirements of the genus remain largely unknown. Seeds are characterised by the presence of a mericarp plug and have fully developed spatulate embryos. In this study, seeds from three species (*Prostanthera eurybioides*, *P. behriana* and *P. calycina*) covering both sections (*Prostanthera* and *Klanderia*) of the genus, were subjected to treatments including pulse dry heat (80°C for 10 minutes), removal of the mericarp plug and combinations of both treatments. Using these approaches, germination rates of 100% were achieved for all species tested. Contrary to earlier reports for the genus, seeds that had been stored under controlled conditions for nearly eight years were shown to be viable and could also be germinated to high levels. Results confirmed that the genus has seeds that are physiologically dormant, and that the mericarp plug plays a significant role in the germination process. Mechanisms by which seed dormancy is alleviated under natural field conditions are proposed.

Keywords: Lamiaceae, *Prostanthera*, germination, mericarp plug, seed dormancy.

Introduction

The genus *Prostanthera* Labill. belongs to the family Labiatae, and is comprised of about 100 known species, all of which are endemic to Australia (Conn 1999). The genus is divided into two sections, sect. *Prostanthera* and sect. *Klanderia* (F.Muell.) Benth., with the division primarily based on floral structure (Conn 1984). Commonly known as mint bushes, many species of *Prostanthera* are recognised in the Australian horticultural industry for their fast growth and spectacular appearance when flowering (Leigh et al. 1984).

Fruits consist of four one-seeded nutlets (mericarps) formed within a persistent calyx, with seeds dehiscing during the warmer months (Toelken 1986). Individual seeds are characterised by a prominent attachment scar that we have defined as a mericarp plug (Ainsley et al. 2008). Whilst the presence of this structure is consistent across the genus, until recently there was limited understanding about the potential role the plug structure may play in the germination process (Ainsley et al. 2008).

In a previous study, seeds of *Prostanthera eurybioides* F.Muell. were found to exhibit physiological dormancy (Ainsley et al. 2008). Contrary to previous reports for other *Prostanthera* species (Sorensen & Jusaitis 1995; Tierney 2006), there was no evidence of seed coat related physical dormancy, and it was concluded that the mericarp plug was acting as a mechanical barrier to ensure *in situ* germination occurs when environmental conditions are conducive to maximising seedling survival. Dormancy in *P. eurybioides* was alleviated using a range of treatments including exposing seeds to gibberellic acid, micro-excision of the mericarp plug, and subjecting seeds to a pulse dry heat treatment. In contrast, the dormancy mechanism and seed ecology for many other species within the genus remains relatively unknown (Carter & Walsh 2006; NSW National Parks and Wildlife Service 2000; Tierney 2006). Knowledge about the germination requirements for other genera of Australian Labiatae appears similarly limited, with only one report for *Hemigenia exilis* (Cochrane et al. 1999) located during an extensive literature search. In that study, the authors reported that it was necessary to remove what was termed a 'seed plug' followed by a chemical treatment with gibberellic acid before seed dormancy was overcome and germination could occur (Cochrane et al. 1999). Therefore the primary objective in the current study was to determine if the germination strategy developed for *P. eurybioides* could be applied to other *Prostanthera* species, and to improve understanding about the dormancy mechanism present in a wider range of species across the genus.

Materials and Methods

Seed material

Three species of *Prostanthera* were tested in this study, two from the section *Prostanthera* (*P. eurybioides* and *P. behriana* Schltdl.) and one from the section *Klanderia* (*P. calycina* F.Muell. ex Benth.). Seeds used for experiments were harvested from wild origin field plants (Table 1). For this study, seeds were harvested between November 2004 and January 2005 depending on the species, and once brought back to the laboratory at the Botanic Gardens of Adelaide in South Australia, stored under constant controlled environmental conditions (15°C and 15% relative humidity) until experiments were commenced during August 2005. These seeds were therefore 7–9 months old when experiments started. For comparative purposes a *P. eurybioides* seedlot harvested in December 1997 that had been dried under ambient conditions, and stored hermetically in dark conditions at 5ºC was also tested. These seeds were 7 years and 8 months old when experiments started. Seeds (Fig. 1) ranged in size (2.5 ± 0.5 × 0.9 ± 0.1 mm), and had fully developed spatulate embryos (Baskin & Baskin 2007). Embryo type was determined by dissecting seeds longitudinally followed by examination under a dissecting microscope. Seedlot viability, as determined by a cut-test on a random selection of 100 seeds, was greater than 80% for all of the seedlots used.

Seed surface sterilisation

To control fungal infestation during germination testing, seeds were surface sterilised in 20% (v/v) hydrogen peroxide (Chem-Supply, Australia) for 10 minutes, then rinsed three times in sterile distilled water. Seed sterilisation was conducted after any pulse heat treatment, and prior to the removal of the mericarp plug to minimise the detrimental affect of exposing moistened seeds to high temperature, and embryos directly to the sterilising solution.

Seed Germination

Based on earlier findings (Ainsley et al. 2008) seeds were subjected to the following treatments: (i) control (no treatment), (ii) pulse dry heat treatment (80ºC for 10 minutes), (iii) excision of the mericarp plug, and (iv) pulse dry heat followed by mericarp plug removal. Heat treatment was applied using pre-warmed convection ovens, with timing of the exposure period commencing once seeds were placed in the oven and the temperature had returned to 80°C. Mericarp plugs were removed by soaking seeds in water for 30 minutes and then excising the mericarp plug using a scalpel blade. Due to the small size of seeds, mericarp plug removal was undertaken using a dissecting microscope.

Experimental design

For all treatments, four replicates of 25 seeds were used. Following any pretreatments, seeds were placed in glass Petri dishes containing dampened white silica sand overlaid with filter paper and incubated under a diurnal temperature regime of 22°C for 12 hours (under light conditions) followed by 10°C for 12 hours (under dark conditions). Light was provided by cool white fluorescent tubes. These conditions are representative of those experienced in the agricultural sector in southern Australia during autumn (Ainsley, unpublished data), and were chosen as they were found to be optimal for the germination of *P. eurybioides* (Ainsley & Ottewell 2008). To maintain moisture levels, Petri dishes were irrigated weekly with sterile distilled water. Germination, defined as radicle emergence ≥ half the length of the seedcoat, was recorded weekly. Experiments were run for 12 weeks, with any non-germinated seeds cut-tested on completion to allow the germination frequency to be adjusted to reflect the actual number of filled seeds tested. Seeds were deemed viable if they were filled with a cream-white and firm endosperm.

Statistical analysis

Final germination totals (recorded at 8 weeks) were analysed for statistical significance by analysis of variance (ANOVA) using SYSTAT 12 for Windows (Systat Software Inc. 2007). To satisfy requirements of normality for statistical analysis, percentage values for germination frequency were arcsine-transformed before analysis (non-transformed data appears in Figures). Fisher's least significant difference ($P < 0.05$) was used to determine whether there were significant differences between treatment means.

Results

For both *P. eurybioides* and *P. behriana* the first evidence of germination for all treatments was observed after 7 days. Where the mericarp plug was left intact, this was characterized by either splitting or lifting of the entire plug structure by the radicle. Radicle emergence was observed 14–21 days after experiments commenced (Fig. 2). In contrast, whilst early germination was also observed for *P. calycina* in seeds where the mericarp plug had been excised, it was not until much later (day 49) that radicle emergence commenced in seeds where the mericarp plug had been left intact (Fig. 2).

Moderate levels of germination (34–52%) were achieved for all three species without the need for pretreatment (Fig. 2). Increases in germination (up

Table 1. Voucher information of seed material used.

Prostanthera eurybioides F.Muell., Mt Monster Conservation Park, S.A. (36º12.221′S, 140º19.330′E), *D.J.Duval 53* (AD172960). — ***P. behriana*** Schltdl., Blackhill Conservation Park, S.A. (34°53.170'S, 138°43.077'E), *P.J.Ainsley 85* (AD173049). — ***P. calycina*** F.Muell. ex Benth., Calpatanna Waterhole Conservation Park, S.A. (33°01.562'S, 134°21.676'E), *P.J.Ainsley 87* (AD173051).

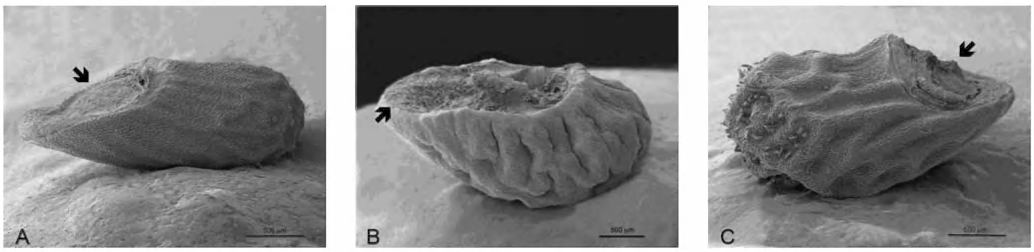

Fig. 1. Seed images. **A *Prostanthera eurybioides***; **B *P. calycina***; **C *P. behriana***. Black arrow indicates locality of mericarp plug. Scale bars: 500 µm. — Seeds were mounted directly onto pin type SEM mounts (12.6 mm diameter) with 12 mm carbon tabs. All samples were coated with gold before observation in a Phillips PSEM 505 scanning electron microscope (Philips, Eindhoven, Netherlands) at an accelerating voltage of 12kV. — **A** AD172960; **B** AD173051; **C** AD173049.

to 35.9%) and rate of radicle emergence associated with a pre-incubation pulse dry heat treatment (80°C, 10 minutes) were found to be significant (F = 18.508, P = <0.001). Removal of the mericarp plug also significantly affected germination totals (F = 105.115; P = <0.001), yielding further increases of up to 2.9-fold for all three species (Fig. 2). The highest level of germination (100%) for all three species occurred by pre-treating seeds with a pulse dry heat treatment (80°C, 10 minutes) followed by removal of the mericarp plug (Fig. 2). Whilst there was no significant difference in germination totals across the species tested (F = 1.881, P = 0.145), a significant interaction between species and the germination treatment (F = 10.252, P = <0.001) was observed. *P. eurybioides* seed that had been stored *ex situ* for nearly eight years germinated to levels similar to freshly collected seed (Fig. 2) with no significant difference in germination observed (F = 0.085, P = 0.773).

Discussion

In this study, high levels of germination (100%) were achieved for all three species of *Prostanthera* being studied. Whilst a moderate level of germination was observed without any pre-germination treatment, the use of pulse dry heat, excision of the mericarp plug or combinations of both these methods, significantly improved germination, and this is consistent with findings that we published from an earlier study (Ainsley et al. 2008). Results also show that similar germination treatments can be applied across both sections of the genus (*Prostanthera* and *Klanderia*) with similar success.

Based on these findings, we propose that non-deep physiological dormancy (Baskin & Baskin 2007) appears to be consistent across the genus and that the mericarp plug plays a significant role in controlling the germination process. Although it has been established through seed imbibition experiments that physical dormancy is not present in *Prostanthera* (Ainsley et al. 2008) the mericarp plug acts as a mechanical barrier and is involved in regulating germination. We concur with the views of Baskin and Baskin (2004), and consider this mechanical barrier a component of physiological dormancy.

For all three species, whilst removal of the mericarp plug maximised germination response, a pulse dry heat treatment was also found to improve germinability. There are a number of potential mechanisms that explain the promotive effect of pulse heat exposures on germination rates and seed dormancy state, including loosening cells in localised regions such as the hilum, chalazal cap or strophiolar plug (Keeley & Fotheringham 1998) and inducing changes to membrane structure (Hallet & Bewley 2002). Whilst it is likely that a pulse heat seed treatment in *Prostanthera* species weaken or loosen cells associated with the attachment point of the mericarp plug, it also appears that other mechanisms within or around the embryo are being affected as seen by the synergistic increase in germination level when combining mericarp plug excision with pulse heat in two of the species (*P. eurybioides* and *P. behriana*) tested in this study.

P. calycina seeds were the largest tested in this study, and are characterised by a larger mericarp plug surface area and thicker seed coat in comparison to *P. eurybioides* and *P. behriana*. These differences in part may explain why it took longer for *P. calycina* seeds with an intact mericarp plug to germinate in comparison to the other species tested. Future research should consider whether this morphological variation relates to a different thermal tolerance range and if a higher pulse temperature for a longer period proves more effective in promoting germination in this species.

The high levels of germination (97%) in eight year old *P. eurybioides* seeds tested in this study imply that this species is orthodox in its seed storage behaviour (Pritchard 2004). Assuming seeds are dried appropriately and stored under suitable conditions, *ex situ* seed storage should provide a viable conservation option. Preliminary data for other *Prostanthera* species that have been stored with reduced seed moisture content (approximately 5%) at low temperature (-20°C) for at least 12 months are yielding similar germination results (Ainsley et al., unpublished). This is in contrast to a previous report (NSW National Parks

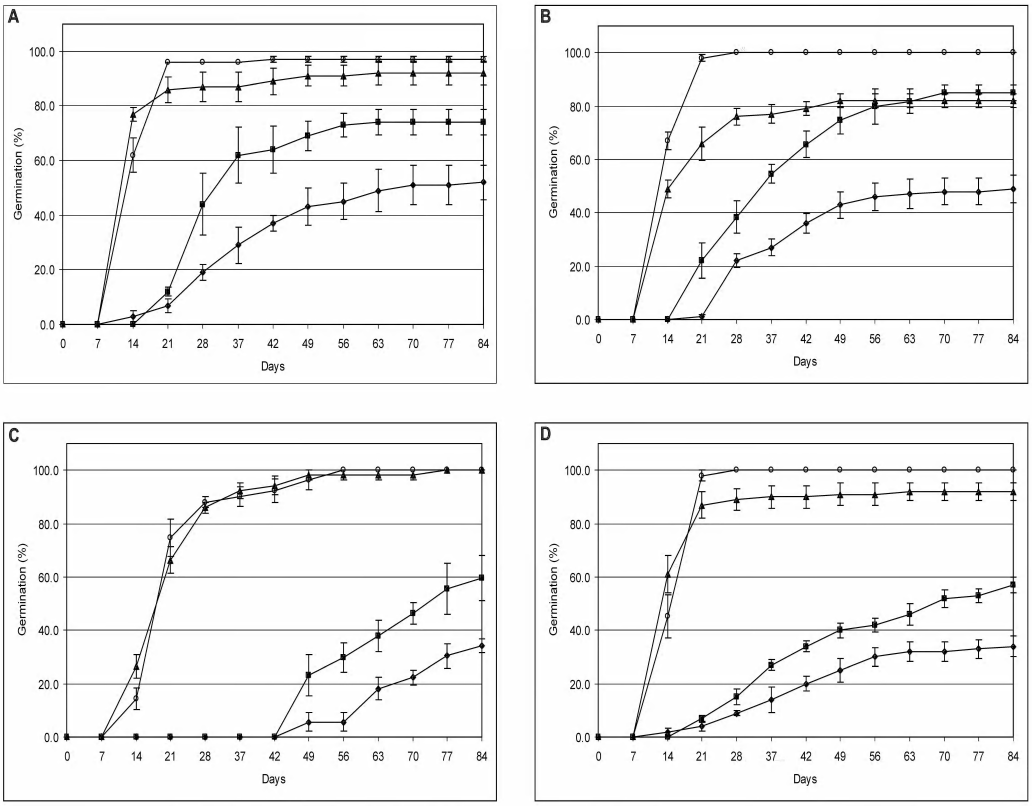

Fig. 2. Germination frequency following various pretreatments (♦ = control; ■ = pulse heat; ▲ = plug removal; ○ = pulse heat + plug removal). **A–B** *Prostanthera eurybioides*: **A** collected 1997, **B** collected 2005; **C** *P. calycina*; **D** *P. behriana*. — Four replicates of 25 seeds were used for each treatment. Seeds were incubated at 10/22°C with a 12 hour photoperiod. Seeds were 7–9 months old when experiments started and had been stored at 15% RH and 15°C. *P eurybioides* seeds collected in 1997 were 7 years and 8 months old, and had been stored at 5°C.

and Wildlife Service 2000) for seeds of *P. junonis* B.J.Conn, which lost all viability within 12-months of collection. The most likely explanation for this contrast is the use of inappropriate storage conditions or poorly interpreted viability results from seeds prior to storage. Unfortunately storage conditions were not described for that study and it is therefore not possible to conclusively resolve this discrepancy.

A number of *Prostanthera* species are considered threatened in their natural environment (Leigh et al. 1984; Carter & Walsh 2006). It is therefore important to improve our understanding about seed ecology in the genus to assist in developing appropriate management plans for conservation significant species. The current study has confirmed that the mericarp plug is a limiting factor in the germination process, and we would like to propose mechanisms by which this could be naturally overcome in the soil seed bank. Under field conditions, surface abrasion or mericarp degradation from soil and sand particles or exposure to elevated temperatures via heat radiation from associated rock surfaces and raised soil temperatures during summer months may assist in this process. The current study also confirms that exposing seeds to moist conditions for extended periods promotes germination in *Prostanthera*. As the three species tested in the current study are endemic to semi-arid southern Australia, which is characterised by limited natural rainfall ranging between 250 and 500 mm per annum, this may be a survival strategy, whereby seeds only germinate in wetter years when the chance of seedling survival and plant establishment is enhanced as a result of prolonged and higher soil moisture levels. There is also evidence that *Prostanthera* seeds respond positively to fire (Carter & Walsh 2006; NSW National Parks and Wildlife Service 2000), including two of the tested species (*P. eurybioides* and *P. behriana*, personal observation). Whilst the exact mode of action is not known (chemical and/or heat), elevated upper soil temperatures experienced during a fire event, which in semi-arid Australian mallee shrublands can range from

60-120ºC to a depth of 5 cm depending on fire intensity (Bradstock et al. 1992; Bradstock & Auld 1995) could induce a physiological response similar to exposing seeds to pulse heat under laboratory conditions.

In Australia many native plant species have complex germination strategies (Merritt et al. 2007), and it is possible that the genus *Prostanthera* may respond to multiple dormancy breaking and germination cues as a natural adaptation to maximise opportunities for germination and enhance population survival in a highly variable natural environment where rainfall and ecological events such as fire are unpredictable and cannot be relied upon.

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